
Modulation of HER2 Tyrosine/Threonine Phosphorylation and Cell Signalling

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1. Introduction

Cell-surface transmembrane tyrosine kinase signalling receptors EGFR, HER3 and HER4 are activated by specific ligand binding, reference in [1] which, induces signalling through intracellular domain kinase-dependant phosphorylation sites, see [2]. The intensity and specificity of the transmitted signal are modulated by homo and hetero-dimerization, allowing other family members to be recruited and thereby amplifying the signal [3]. HER2, which does not have a defined activating ligand, plays a key role in its capacity to amplify the signal [4] by being the preferred partner for hetero-dimerization. Both HER2 hetero and homo dimers maintain their intracellular kinase and phosphorylation sites in an activated state [3,5,6,]. HER2 hetero and homodimers both maintain their intracellular kinase and phosphorylation sites in an active state [5,6] Dimerization with HER2 increases receptor-ligand affinity and receptor-ligand stability, further enhancing activation [4,7]. In vivo enzymatic processing releases the HER2 extra-cellular domain (95 kD), including the dimerization site [8] from the membrane, but the role this plays in signal modulation remains undefined [9]. HER2-dependant activation signals promote a highly phosphorylated tyrosine state in the intra-cellular domain, which is then recognized by a family of specific cytoplasmic signal transducers [10]. These signal transducers regulate cell proliferation, apoptosis and cell characteristics associated with the transformed state [1,2 5,6]. Mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-OH kinase (PI3K- AKT) pathways [11,12] have been particularly well studied as downstream signal pathways of HER2 leading to the concept of an oncogenic unit [13]. Accumulating experimental and clinical data link anti-HER2 treatment to inhibition of these downstream pathways suggesting that they are critical mediators of the activated HER2 state and cell survival [14]. However, whether the trastuzumab mediated inhibition of signalling is due to reduced HER2 phosphorylation/kinase activity, inhibition of HER2 dimerization, decreased HER2 levels, altered metabolic processing of HER2, binding dependant allosteric effects on HER2 or HER2-HER1/3/4 or a combination of these mechanisms is unknown.

In most cases, amplification of HER2 in breast cancer is associated with an amplicon comprising genes mapped to the 17q12-q21 region [15-18] that includes several genes involved with cell proliferation [16], transformation [17], adhesion [16] and chemotherapy sensitivity [18]. Clinically, the amplicon gene copy number is a parameter closely linked to trastuzumab and lapatinib efficacy [19,20].

Experimental evidence now suggests that nuclear localization of growth factors and receptors is not uncommon and also occurs with members of the EGFR family [21]. More recently, specific products derived from the HER2 receptor have been shown to have independent gene regulatory effects by virtue of a nuclear localization motif encoded in the HER2 protein [21,22] and by alternate splicing of the HER2 mRNA [21]. For example, the nuclear localization sequence preferentially targets a HER2 derived peptide to bind to sequences, one of which is in the COX-2 promoter region, leading to up-regulation of COX-2 expression [22]. Whether there are other HER2 derived peptides and the role they may play in the signalling cascade and whether any of the current anti-HER2 treatments modulate these factors remains is an area of current interest.

The original anti-HER2 mouse monoclonal antibody, 4D5, was shown to inhibit HER2 tyrosine phosphorylation [23] and breast cancer cell proliferation [23]. This antibody was humanized through genetic engineering to become trastuzumab [24,25]. A second antibody now in development (pertuzumab) binds to an extra-cellular HER2 epitope in close proximity to the dimerization site and has been shown to block dimerization [26-, 28]. A small molecule HER2/1 tyrosine kinase inhibitor, lapatinib has shown single agent activity in the clinic [20,29,30], providing further evidence that the disruption of the EGFR1/HER2 activation pathway, specifically by blocking phosphorylation [29-32], leads to cell death.

The focus of this investigation was to examine the effects of trastuzumab and lapatinib on breast cancer cell lines containing the HER2 amplicon under conditions of steady state anti-HER2 growth inhibitory effects. The quantifiable end point was the inhibition of proliferation as determined by MTT and Tritiated thymidine incorporation. These assays were used as evidence that the trastuzumab or lapatinib treatments had interrupted signalling pathways. Using immune detection of three distinct HER2 protein sites, four distinct HER2 tyrosine phosphorylation (P-Tyr) sites, a threonine phosphorylation (P-Thr) site and actin, changes in these parameters were monitored during anti-HER2 treatment. The demonstration of the restricted tyrosine kinase inhibitory activity of lapatinib further validated the specificity of the anti-HER2 phospho-tyrosine/threonine (P-Tyr/ P-Thr) antibodies selected for this investigation.

2. Materials and methods

Breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) specifically for these experiments and maintained in RPMI 1640 with 50 units penicillin/streptomycin, 2 mM glutamine, 7.5 % fetal bovine serum, and incubated at 37° C in 5% CO₂. The ATCC authenticates and tests cell lines provide for research as per their protocols.

Four drug treatments and a control, done in quadruplicate, were analyzed using Western blot techniques. Cell concentrations were adjusted to 375,000 cells/ml in complete media and 1 ml was added to each well of a 12 well tissue culture plate. Cells were allowed to adhere to the plates for 24 hrs. Drugs or control solutions were added at a final concentration of 100 microgram/ml trastuzumab, (Herceptin) or control rituximab (Rituxin), (Genentech, San Francisco, Ca). Preliminary dose finding experiments were carried out at 2-250 micrograms/ml. 0.1% DMSO and 0.1%DMSO + 10 nM Lapatinib (Tykerb) (GlaxoSmithKline Research Triangle Park, NC) was also tested following this same procedure .The cells were incubated at 37° C in 5% CO₂ for 8 or 48 hrs. The cells were lysed with either 400 µl of SDS loading buffer (58 mM Tris 6.8, 1.6 % SDS, 6% glycerol, 0.83% BME, 0.002% bromphenol blue) or NP-40 (0.5% NP-40, 50 mM Tris 7.4, 120 mM NaCl, 2.5 mM EGTA). Additional cell growth inhibitory studies were carried out for incubations up to 9 days in 6-well plates, and cells were tested serially every other day by pulse Tritiated thymidine incorporation and MTT assays

For Western blotting, samples were boiled in electrophoresis sample buffer containing 0.0625M Tris-HCl (pH6.8), 10% glycerol, 2% SDS and 5% BME, for 10 minutes then separated on 12% SDS-PAGE gels (mini-protean, Bio-Rad, Richmond, CA) at 100 V until the dye front reached the bottom then transferred overnight (47 mA) to nitrocellulose membranes (Bio-Rad). Membranes were blocked with a 5% w/v solution of non-fat dry milk in TPBS for 2 hours at room temperature (RT). The blots were then probed with primary antibodies according to the respective titers provided by their manufacturers and the membranes were rocked for 2 hours at RT. The blots were washed 1X with TPBS and a 1:10,000 dilution of secondary antibody (either goat anti-rabbit alkaline phosphatase or goat anti mouse alkaline phosphatase (Sigma, ST Louis, MO) was added, and the blots rocked at RT for 1 hour. The blots were washed 3X with TPBS and the membrane developed in the alkaline phosphate substrate NBT/BCIP (Promega Corp., Madison, WI).

Rabbit anti-P-Tyr 877, P-Tyr 1112, P-Tyr 1221/1222, P-Tyr 1248 P-Tyr 877, P-Tyr 1221/1222, and anti-amino acid peptide containing residue 1222, were purchased from Cell Signalling Technology, Inc.(Danvers, MA). Anti-P-Tyr 4G10, was purchased from Millipore, (Billerica, MA). Anti-extracellular HER2, Neu (9G6):sc-08, anti-HER2 P-Thr 686, p-Neu (7F8):sc81508, anti-HER2 P-Tyr 1112, p-Neu (19G5):sc-81528, anti-extracellular HER2, Neu(ER23):sc-74241, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HER2 (AA 7570-987) was purchased from ProMab Biotechnologies, Inc. (Albany, CA). All antibodies were used per the manufacturer's recommendations. Membrane fractions were isolated using the Perfect-Focus Membrane Protein kit, (G Biosciences, Maryland Heights, MO), according to the manufacturer's protocol.

Human serum samples from patients and normal volunteers were collected under an Institutional Review Board approved research protocol and stored in our serum bank of de-identified serum specimens, stored at -78 degrees Celsius.

3. Results

3.1. Cell growth-inhibitory and viability assays

To define the experimental time points where anti-HER2 therapy modulation of downstream signalling could be demonstrated, breast cancer cell lines were tested for trastuzumab/lapatinib growth inhibitory effects by Tritiated thymidine incorporation and MTT assays. Three HER2 amplicon-containing cell lines were examined, BT-474, SK-BR-3, MDA-MB-453, and amplicon (-) MCF7 served as a control [29,31]. Cells were treated in log phase growth under conditions where factors such as confluence inhibition were minimized. Antibody controls included isotype matched human/mouse IgG1 chimeric (rituximab) and fully humanized IgG1 antibody (bevacizumab). In addition, IgG1 cetuximab, which blocks EGFR1 signalling, was used as an additional control; as in a separate set of experiments, we confirmed that bit did not induce a proliferation or inhibitory state in these cell lines, in agreement with reports by others, nor did we find any effect on HER2 tyrosine phosphorylation [29]. Trastuzumab was tested at a continuous cell exposure dose of 100 micrograms/ml with select experiments carried out at 2- 250 micrograms/ml. Cell cultures were assayed at various time points up to 9 days of treatment. Results of cumulative and pulsed-chased Tritiated thymidine incorporation and MTT assays were in agreement, demonstrating a reproducible inhibitory effect of trastuzumab on cell growth and division. This is best demonstrated by the cell line BT-474. Cell lines SK-Br-3 and MDA-MB-453 exhibited lesser effects and no effect was seen on HER2 amplicon negative control MCF7 cells. These results are consistent with previous reports [29,31]. The Tritiated thymidine incorporation experiments suggested that a trastuzumab-mediated continuous cell-growth inhibitory state is established throughout the incubation period by demonstrating reduced incorporation per cell at each time point tested in the trastuzumab treated cultures (Table 1). Significant cell death was not observed with trastuzumab up to the 9-day time point. Of note was that the maximum cell-growth inhibition by both MTT and Tritiated thymidine incorporation assays was observed at 5 micrograms/ml of trastuzumab, thus the assays described below were carried out at 20-50 times the active biological dose. The 100 micrograms/ml dose was selected as it most closely matches doses achieved in patients.

Effective doses for lapatinib in the cell culture assays ranged from 1 nanogram/ml to 1 microgram/ml. In contrast to results with trastuzumab, lapatinib induced significant cell death by day five (> 90%) under conditions of continued cell exposure at a constant drug dose of ≥ 10 nanograms. Lapatinib cell cultures beyond five days had no measurable MTT signal, lack of Tritiated thymidine incorporation (< 1% initial values) and few viable adherent cells by microscopic examination. Experiments with lapatinib included a dimethyl sulfoxide (DMSO) control, as it was the solvent used for this lipophilic drug. Control DMSO had a small effect on growth kinetics but no cell death was observed. In order to examine the comparative biologic/phosphorylation inhibition effects of these two agents, time points of 8 hours and 48 hours were selected for further analysis. The critical feature of these two time points is that cell viability appeared high in both trastuzumab and lapatinib cultures as

demonstrated by the MTT assay, Tritiated thymidine incorporation, protein levels of HER2 and actin in cell cultures as assessed by Western blots, and the continued adherence of cells up to 48 hours as determined by microscopic examination. These experiments would therefore allow for the examination of molecular events prior to cell death, distinguishing between events that may be related to induction of cell death versus changes resulting from cell death.

TRASTUZUMAB	DAYS	1	3	5	7	9
% ³ H-thymidine incorporation	Cell lines					
	BT474	76.3	81.5	76.7	68.5	66.2
	SK-BR-3	84.4	80.2	80.4	77.6	65.1
	MDA-MB-543	81.4	84.3	78.3	73.9	67.7
	MCF-7	98.7	97.9	101.6	99.0	100.7
% MTT						
	BT474	99.3	95.4	87.6	62.1	45.4
	SK-BR-3	97.7	93.6	75.5	70.1	65.3
	MDA-MB-543	99.7	97.3	90.3	82.1	71.2
	MCF7	97.7	99.8	102.2	100.6	101.0
LAPATINIB	DAYS	1	3	5	7	9
% ³ H-thymidine incorporation	Cell lines					
	BT474	80.3	77.2	6.6	1.1	----
	SK-BR-3	84.7	82.1	8.8	----	----
	MDA-MB-543	88.7	82.0	8.4	----	----
	MCF-7	102.0	101.2	96.7	98.5	97.9
% MTT						
	BT474	97.6	88.4	4.7	----	----
	SK-BR-3	99.9	96.8	14.3	----	----
	MDA-MB-543	100.5	97.9	13.6	----	----
	MCF-7	104.1	97.9	97.6	101.7	100.6

Values are expressed as %-treated cells/controls for each time point. Data shown represents experiments carried out with trastuzumab 100 micrograms/ml and lapatinib 10 nanograms/ml While the MTT assay represents relative total viable cell counts of treated cells compared to controls, the ³H-thymidine counts/minute (cpm) were corrected for number of viable cells using the MTT assay data (cpm treated/cpm control/MTT treated/MTT control). The paired data sets were evaluated by Student's t-test for significance and those values achieving statistical significance are **bolded**. --- indicates no measurable signal.

Table 1. Relative growth inhibitory values

3.2. Western blot experiments

3.2.1. *HER2 protein levels*

Protein level changes for HER2 and actin, associated with trastuzumab or lapatinib treatment at two selected treatment time points (8 and 48 h) were analyzed by Western blot analysis. Three specific polyclonal antibody sera detecting HER2 were tested, one recognizing epitopes in the ectodomain (amino acid 182-373), and two detecting epitopes in the cytoplasmic domains (amino acids 750-987 and a determinant in proximity to the Tyr 1222 amino acid). These experiments were carried out at least four times each and representative panels of a single experiment are shown in Figure 1. The three controls shown here were cells grown in media alone, cells grown in the presence of rituximab, and cells grown in DMSO. All controls gave very similar bands for the three anti-HER2 antibody sera. Small changes in HER2 levels are first detected at 48 hours in cell line SK-Br-3 treated with lapatinib, while actin levels remain unchanged at this time point. This finding is consistent in each of the four separate experiments and confirms the increase in cell surface HER2 levels associated with lapatinib treatment as previously reported for cell line SK-Br-3 [31]. This time point is just prior to the onset of events associated with apoptosis and may represent a consequence of cellular pathway disruption, as increased HER2 mRNA levels were not observed. In separate experiments, cells grown in the presence of cetuximab or bevacizumab (not shown) resulted in western blots identical to controls indicating a lack of modulation of HER2 protein expression by anti-EGFR or VEGF therapy in these cell lines. These results are consistent with our previously reported analysis of EGFR, HER2, HER3 and HER4 mRNA levels by gene expression mRNA array analysis of mRNA extracted from these breast cancer cell lines treated with trastuzumab compared to these controls [30]. These parallel experiments used extracts from these breast cancer cell lines treated with trastuzumab or cetuximab, or control antibodies, under these same defined conditions and time points.

No difference in actin levels was noted in each set of these experiments. As the cell count number in the first 48 hours showed <10% difference, the amount of cell extract applied to the gel while corrected for cell number at each time point had very little variance. Thus, these results are consistent with those of the Tritiated thymidine and MTT assays indicating the minor growth inhibitory effects of these agents up to 48 hours. These finding also support the contention that biologic events occurring up to 48 hours of treatment precede cell death events and are possible activators of critical pathways leading to cell death.

3.2.2. *Tyrosine and threonine phosphorylation levels in the control cultures:*

Four site specific anti-P-Tyr antibodies (Tyr 877, 1112, 1222 and 1248) and one anti-P-Thr (Thr 686) were tested on cell extracts collected at specific timed intervals (Figure 1). The controls showed consistent pattern of staining at the two time points examined for each cell line, but different patterns of relative intensity for each tyrosine or threonine were observed for each cell line. These results suggest that each cell line has a distinct net P-Tyr signalling oncogenic unit, rather than a HER2 specific net down-stream signalling motif.

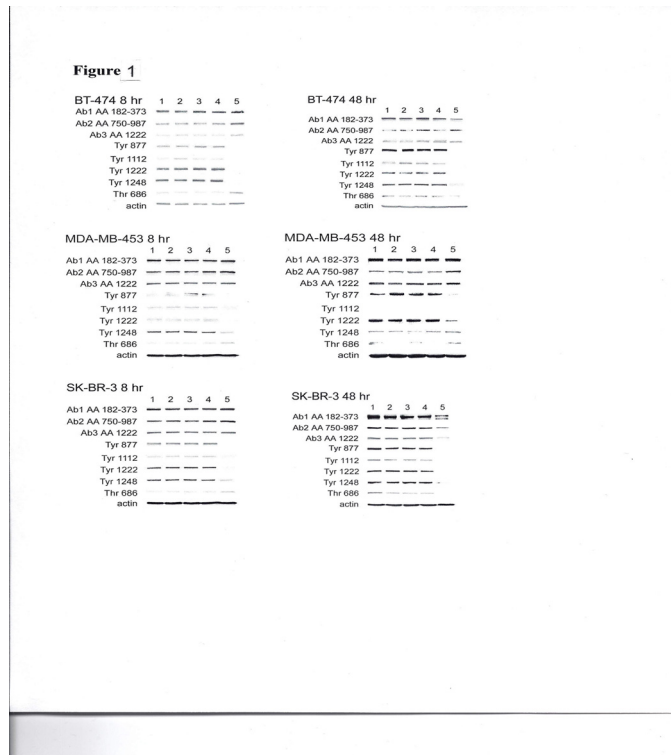


Figure 1. Shows Western blots bands of HER2, phosphorylated tyrosines/threonines, and actin from representative experiments of three cell lines growing in the presence of trastuzumab or lapatinib, or their controls, tested at 8 hours and 48 hours after drug administration. Primary antibodies were used at 1:5,000 for anti-P-Tyr/Thr and anti-HER2 antibodies and 1:10,000 for anti-actin antibodies and secondary antibodies. Labelled lanes show trastuzumab-treated cells in *lane 2* with its controls in *lane 1* (media alone) and 3 (control antibody). *Lane 5* shows lapatinib-treated cells and the control grown in lapatinib solvent, (DMSO) in *lane 4*. Blots show the p185 band with secondary reagent as shown, and actin is presented as proof of viability and equivalent cell number applied to each *lane*. These gels demonstrate the contrast in phosphorylated tyrosines detected comparing lapatinib to trastuzumab and confirm the TKI activity of lapatinib. At 8 hours, lapatinib almost completely blocked tyrosine phosphorylation with virtually no discernible difference in the in the HER2 protein levels. The 48 hour time point was selected as it represented the last time point tested prior to lapatinib-induced cell death, which was almost complete by day 6.

Compared to the media controls, the DMSO controls did not differ in phosphorylation levels of these specific P-Tyr or P-Thr. While the intensity of the bands were similar for each cell line at each of the two time points, exceptions were noted for Tyr 877 and cell lines BT-474 and MDA MD 453, where phosphorylation is clearly increased from the 8 hour to the 48 hour time point. Similarly, P-Tyr 1222 also demonstrated increased intensity from 8 to 48 hours in cell line MDA-MB-453. Whether these findings suggest that the cells are not completely recruited into log phase growth at the 8-hour time point or cell-cell contact plays a role is unclear. The actin and HER2 bands strongly suggest equivalence of cell number applied to each lane at each of these time points.

3.2.3. Tyrosine and threonine phosphorylation levels in trastuzumab and lapatinib treated cells

No discernable change in phosphorylation of the P-Tyr or P-Thr was detected for trastuzumab-treated cells despite ongoing inhibition of cell growth at these time points. The unexpected lack of trastuzumab inhibition of tyrosine phosphorylation prompted experiments that replaced fetal calf serum (FCS) with human serum to determine if a component of FCS was responsible for inhibiting the full trastuzumab activity. Cells grown in media with 10% human serum from breast cancer patients or normal females, pre or postmenopausal, or male serum did not affect these findings (data not shown). Doses of trastuzumab up to 250 micrograms/ml did also did not change the patterns of phosphorylation. In contrast to the results demonstrating constant HER2 protein steady state levels, P-Tyr levels in lapatinib treated cells are markedly reduced (Tyr 1248) or absent (Tyr 687, 1116 and 1223), consistent with its role as a HER2 tyrosine kinase inhibitor (Figure 1). In addition, the P-Thr level is not affected by lapatinib exposure, demonstrating the tyrosine backbone specificity of lapatinib. As the lapatinib Western blots demonstrate, HER2 and actin protein levels are maintained through 48 hours, these results strongly suggests that changes in detected phosphorylation levels are due to kinase inhibition rather than the substantial in HER2 protein or cell death. We suggest that these early findings are related to the lapatinib mechanism of cell death that is apparent 2-3 days later.

3.3. MRNA expression patterns in trastuzumab treated breast cancer cells

3.3.1. Detection of a small molecular weight HER2-like product

Cell fractionation experiments were carried out to determine whether HER2 derived products with nuclear localization motifs could be identified by Western Blot analysis and to determine if trastuzumab or lapatinib altered their molecular processing mechanism. Differential generation of enzymatically derived nuclear localizing products could be responsible for trastuzumab mediated trans-membrane growth inhibitory effects. The results indicate that these techniques were not sensitive enough to detect, or were not of the correct immune-specificity to detect HER2 derived product in concentrates of nuclear or cytoplasmic cell fractions (data not shown).

However, using the anti-HER2 external domain antibody (AA 182-373), we detected a small molecular weight (20 kD) external domain-like HER2-derived product associated with the cell (Figure 2). This peptide segment is too far from the hydrophobic transmembrane segment to contain a transmembrane anchoring component to explain its association with the cell pellet, as it is too small (approximately 180 amino-acids in length) to extend from position 373 to the transmembrane domain. This peptide was not detected in supernatant or concentrates of supernatants. Cells exposed to acid conditions (pH 4.0, 0.5 M acetate, 0.15 N NaCl) to elute this 20 kD segment from the cell surface demonstrated that the 20 kD peptide could not be eluted under these conditions and was still found associated and concentrated

in the cell pellet fraction (figure 2). Because concentrates of cytoplasmic fractions and nuclear fractions did not demonstrate the presence of this 20 kD peptide segment, a Triton X-114 fraction of the cell membrane was assayed and found to have high levels of this product (Figure 2). These results suggest that this product is derived from the extra-cellular domain of HER2 by enzymatic processing and binds avidly to a cell membrane-anchored determinant or is internalized into a sub-membrane compartment, co-purifying with the membrane fraction, or that it is a membrane protein cross reactive with antibody to extracellular domain (AA 183-373). The significance of this finding is that this peptide appears to be inhibited by lapatinib treatment while the intact HER2 levels remain constant and expression of this protein is unaffected by trastuzumab treatments (Figure 2). Thus, this small molecular weight product may be related to the unphosphorylated state of HER2.

4. Discussion

HER2 transmembrane activation signals promote a high level of selected intracellular domain P-Tyr sites, which are then recognized by specific cytoplasmic signal transducer molecules [10]. Thus, HER2 acts as an oncogenic unit transmitting cell survival, growth, and cell proliferation signals to critical intracellular pathways such as Mitogen-activated protein kinases (MAPKs), AKT and phosphatidylinositol 3-OH kinase (PI3K) pathways[11,12,29]. Here, we show that the relative level of specific P-Tyr is different for each HER2 amplicon-positive cell line examined, and thus, each oncogenic unit may be distinct in each tumour [13]r. Linkage of anti-HER2 treatment to the inhibition of these downstream pathways is well established, suggesting that these specific P-Tyr are critical mediators of the downstream signalling effects of the activated HER2 state and cell survival [7,10-12,29,30]. The molecular mechanism by which trastuzumab, a humanized IgG1, modulates the intracellular tyrosine kinase site from its extra-cellular binding site has remained unanswered. For example, cetuximab binds to, or near, the ligand binding site of EGFR thus acting as an antagonist [1,2,30-32]. Defining the molecular mechanism by which trastuzumab mediates its anti-HER2 effects could reveal a general non-ligand site-specific strategy for development of receptor inhibitory monoclonal antibodies in other receptor kinase systems.

The primary focus of this investigation was to determine the key molecular mechanism(s) governing trastuzumab's transmembrane modulation of downstream effects. Here, we show that trastuzumab binding does not appear to alter the net steady state levels of p185 HER2 protein as detected by all three of the anti-HER2 antibodies tested. Previous gene mRNA expression array findings demonstrate that no significant change in mRNA levels for HER2 or the other members of the ERBB family were induced by trastuzumab binding [33]. Taken together, these results strongly argue against an antibody-dependent alteration in natural turnover rate or internalization kinetics of HER2 [34]. We conclude that in the absence of demonstrable modulation of HER2 membrane mass on the cell surface, HER2 protein synthesis, and thus, alteration of kinetics of internalization or HER2 metabolism, net

membrane HER2 levels could not be implicated as a mechanism to down-regulate HER2 signalling. Despite these observations for HER2, our gene mRNA expression array data did suggest numerous intracellular pathway modulations mediated by trastuzumab binding to the target cells examined here [33]. These findings confirm transmembrane signal transduction modulation directed and specific to trastuzumab binding [33-35]. For example, we found that, the ubiquitin pathway is up-regulated upon trastuzumab binding to HER2 [35], as has been reported by others through ligase activity at tyrosine 1112 [35,36].

Trastuzumab has not been definitively shown to retain the tyrosine kinase inhibitory activity of the parent 4D5 mouse monoclonal antibody [23]. Here we show that the level of HER2-P-Tyr/Thr corrected to cell number, actin levels, and the steady-state levels of intact extractable HER2, is not altered by exposure to trastuzumab. The finding that both P-Tyr levels and HER2 levels in each of these assays reveal no change further supports the conclusions derived from the gene mRNA expression array data [33]. Thus, examination of HER2 steady-state protein levels and key phosphorylation sites [10] did not reveal the molecular perturbation responsible for well-documented trastuzumab inhibition of cell proliferation and downstream pathways.

In contrast to trastuzumab, lapatinib treatment almost completely abrogates the HER2-P-Tyr as a signalling element while leaving HER2 protein levels unchanged. Thus, lapatinib acts through the blockage of tyrosine phosphorylation rather than the modulation of internalization or metabolism of the HER2 protein. We further show a sequential relationship between lapatinib-induced blockage of HER2-P-Tyr (8 and 48 hours) and induction of cell death at day 5-6 of drug exposure. At this late time point, MTT, Tritiated thymidine, actin levels and microscopy all support a lapatinib induction of cell lyses.

The inhibition of cell growth was used to define the conditions of ongoing drug-induced biological effects. The cell growth experiments described here are in agreement with similar reports by others [24,31]. Trastuzumab induced slower growth but did not suppress net increases in cell numbers over time. This *in vitro* effect of trastuzumab parallels the clinical observation that a prolonged stable disease is a common outcome of treatment [37].

Treatment of amplicon-positive breast cancer cells with cetuximab confirms reports by others that anti-EGFR therapy does not inhibit proliferation, induce cell death, nor inhibit HER2 phosphorylation in these cell lines [31]. Furthermore, we did not find that combinations of cetuximab and trastuzumab augment inhibition of cell growth or promote apoptotic events [31]. Cell growth inhibition and phosphorylation experiments with cetuximab have not been in agreement with results of similar investigations using small molecules with EGFR tyrosine kinase inhibitory activity (TKI) [38,39]. However, drugs such as gefitinib, which are believed to be relatively specific for EGFR, are also found to block HER2 phosphorylation at higher doses [38], complicating the interpretation of its mechanism of action [38]. Whether there is an independent anti-EGFR activity cross-inhibiting HER2 phosphorylation (cross-talk) or whether this observation is a consequence of the direct anti-HER2-phosphorylation activity of gefitinib is unclear. Results of these *in*

vitro experiments may mislead investigators into attributing findings to cross-talk between receptor pathways rather than cross-reactivity of the reagent tested. We may over estimate biologic activity when reagents are used at too high of a concentration or too long of an exposure. Results of these experiments may mislead investigators into attributing findings to cross-talk between receptor pathways rather than the cross reactivity of the reagent tested. Thus, we conclude that results of experiments testing cetuximab's biologic effects more closely represent, and are more specific to, the pure anti-EGFR blockade. We clearly show here, in agreement with previous reports by others that pure anti-EGFR1 inhibition of signalling does not result in cell death or modulation of HER2 phosphorylation levels [29].

These observations suggest that the anti-EGFR1 tyrosine kinase effects of lapatinib are not contributing to its anti-HER2 effects. Thus, the results of the panel of anti-HER2-P-Tyr-antibodies demonstrating almost complete abrogation of HER2 phosphorylation by lapatinib and its associated delayed cell cytotoxicity (day 5-6) establishes a specific and unique link between HER2 tyrosine phosphorylation and cell survival. Importantly, these results also suggest that additional signalling through HER3 and HER4 may not be critical for apoptosis [40], except as it may ultimately be mediated through HER2 tyrosine kinase inhibition (reduction in hetero-dimerization) [40]. Thus, blocking dimerization by itself, which is unlikely to affect steric change in the intracellular domain, will not induce a modulation of P-Tyr levels. Taken together, these data suggest that testing the effect of a specific and robust HER2 tyrosine kinase inhibitory reagent is warranted.

In the clinic, where the anti-EGFR toxicity of lapatinib is dose limiting (diarrhea and skin rash) and prohibits dose escalation, the testing of a small molecule TKI manifesting pure HER2 kinase inhibition would be of great interest. Recently a pan ErbB TKI was tested and shown to be dose limited by the EGFR-mediated bowel toxicity, further supporting the conclusions presented here [41]. A pure anti-HER2 agent would be even more compelling if one could demonstrate upon dose escalation increased tumor cytotoxicity and a lack of other intervening non-HER2 related dose-limiting toxicities. Close examination of many of the TKIs demonstrate widespread promiscuity with many unexpected cross-reactivities [42]. In general, clinical success of a widely reactive kinase inhibitor is not informative as an identifier of the key pathway(s) responsible for mediating anti-tumor effects and often leads to "assumed" mechanisms of activity.

Using the four anti-P-Tyr sera, we could not find any diminution in the phosphorylation levels as assayed by Western blots, in cells treated up to nine days in the presence of trastuzumab. In addition, the anti-phospho-threonine reagent was used as a negative control, and was found not to be blocked by lapatinib demonstrating the specificity of the drug and the kinase activity. Thus, we propose that the kinase inhibition that was originally described by the mouse antibody 4D5 [23] has been lost in the humanization process [25]. The abundance of well-documented evidence that inhibition of specific downstream pathways is a consequence of trastuzumab-cell binding remains unexplained.

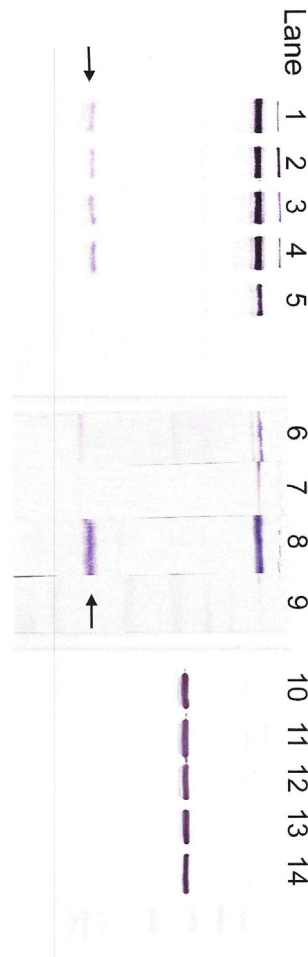


Figure 2. Demonstrates a series of experiments evaluating a 20 kD protein detected by anti-HER2 extracellular domain (AA 182-373) anti-sera at 48 hours. *Lanes 1-5* demonstrate the full Western blot gel of BT-474 (48 hours, from Figure 1) showing two bands: the p185 HER2 protein and the 20 kD protein (arrow on left) *lanes 1-4*. Note that this protein is not detected in *lane 5* which represents lapatinib-treated cells, while the p185 HER2 is mildly reduced in *lane 5*. *Lanes 1-14* show the corresponding actin control confirming that equal numbers of cells were applied to *lanes 1-5*. Exposing BT-474 to cells to an acid wash of pH 4.0 does not elute the protein as *lane 7* is the acid wash and the cell pellet that remains is shown in *lane 6*, has both p185 and the 20 kD protein. Using the Triton X Perfect Focus Membrane Protein kit methodology shows a clearer picture of localization of the 20 kD band with membrane HER2 protein and the 20 kD band (arrow) both in *lane 8* (membrane protein concentrate) and corresponding cell pellet, run in *lane 9*, is negative.

One hypothesis to be considered is that the trastuzumab effect is a weaker version of what is seen with lapatinib and while we cannot demonstrate a small loss of P-Tyr activity in culture, more sensitive techniques might be informative. Alternatively, trastuzumab inhibition may be allosteric or conformational in nature and at the level of the cytoplasmic signal transducers [10] ability to detect the phosphate moieties in the context of an altered peptide conformation [43]. One may further speculate that due to the unique binding epitope of trastuzumab, which is in extra-cellular proximal domain, trans-membrane tertiary structural changes may be induced. Cross-linking HER2 (dimers) at the extracellular proximal domain may disrupt the P-Tyr access to cytoplasmic signal transducers by physical constraints. This hypothesis would suggest that other membrane receptor systems could also be modulated in a similar fashion.

Our results demonstrate the presence of a small 20 kD protein concentrated in the extracted membrane fraction which is immunologically similar to an extra-cellular segment of HER2 protein. This may provide further evidence of specific enzymatic digestion of HER2 into novel biologic agents [44,45]. The low molecular weight of this identified fragment and its association with the membrane fraction raises the possibility that it may function as a ligand for another, as yet unidentified, receptor. Thus, we hypothesize that HER2 may not be a classical receptor as has been suspected by the lack of a ligand binding moiety but rather a pre-ligand entity which requires regulated and specific digestion to be activated. The differential expression of this moiety with lapatinib in contrast to trastuzumab treatment, demonstrates the complexity of this system and the difficulty in attributing a single molecular mechanism in transmembrane signal transduction.

These observations are not informative regarding the precise cytotoxic mechanism of trastuzumab activity in the clinic. Models based on preclinical animal studies favor immune mechanisms as key in mediating tumor shrinkage [46]. Studies of patients treated in the neo-adjuvant setting also support these findings [43]. However the clinical effects of trastuzumab could be divided into two distinct effects, a growth inhibitory effect as seen in vitro and which manifests clinically as long term stable disease and a cytotoxic effect as one might expect from immune mediated cell lysis which results in rapid tumor shrinkage in the clinic, especially as seen in neo-adjuvant studies [47].

In addition to these hypotheses, there is evidence that HER2 induced downstream effects actually amplify pro-inflammatory factors such as COX-2 [22] creating a micro-environment for promoting immune cell mediated tumor cell killing. These data are in agreement with modulation of a prostaglandin pathway identified by gene mRNA expression array experiments [33] in trastuzumab treated cells. Thus downstream events induced by HER2 or regulated by trastuzumab binding, may promote the immune mediated killing of tumor cells by trastuzumab. These findings provide a strong rationale for combining other anti-breast cancer cell antibodies with trastuzumab therapy. Furthermore the additional anti-tumor efficacy observed with combinations of trastuzumab and chemotherapy could also be attributable to the known effects of certain chemotherapeutic agents, which render tumor

cells more sensitive to immune attack [48]. If this is the case, research should be focused on mechanisms by which chemotherapy may damage tumor cells sufficiently to allow more efficient killing by immune mechanisms rather than focus on apoptotic pathways that enhance chemotherapy cytotoxic effects.

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Disclosure statement

The authors declare that there are no competing financial interests in carrying out this study.

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